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Natural interspecies transfer of mitochondrial DNA in amphibians

(introgression/restriction enzymes/*Rana*/phylogeny/evolution)

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ABSTRACT **mtDNAs of two Central European water frog species, *Rana ridibunda* and *Rana lessonae*, were examined by electrophoresis of restriction enzyme fragments. Two types of mtDNA occur in *R. ridibunda*. One shares with mtDNA of *R. lessonae* 25.8% of 132 fragments generated by 19 enzymes, corresponding to a nucleotide sequence divergence of 8.1%; the other has diverged from *R. lessonae* mtDNA by only 0.3%. This latter type is a variant *R. lessonae* mtDNA that has been transferred into *R. ridibunda*; the introgression may have occurred via the hybridogenetic hybrid lineages collectively known as *Rana esculenta*. Of 37 *R. ridibunda* from Poland, 59% had the typical *R. ridibunda* mtDNA; 41% had the modified *R. lessonae* mtDNA as did a single individual from Switzerland (introduced). A single *R. ridibunda* from Turkey, outside the present range of *R. lessonae*, had the typical *R. ridibunda* mtDNA phenotype. Discordancies between inheritance of mitochondrial and nuclear genomes point up the danger of relying on a single molecular feature in reconstructing phylogeny. In addition, studies of mtDNA provide otherwise inaccessible information on complex evolutionary histories of closely related species. A knowledge of these complexities is important to an understanding of phylogenetic relationships and of the genetic processes that underlie the evolution of clonal taxa.**

Determination or estimation of sequence differences of mtDNA is a powerful tool for reconstructing the genealogy or phylogeny of closely related groups of individuals, populations, or species. Since mtDNA is maternally inherited (1–4), it is an excellent marker for identifying the maternal parent in hybridizations giving rise to parthenogenetic (5, 6) or gynogenetic (unpublished data) species. In addition, mtDNA analysis has been useful in determining relationships both within (7–12) and among (13, 14) species. Both a strength and a weakness of mtDNA analyses, however, is that genealogies revealed by them reflect only the maternal histories. Furthermore, phylogenies of species reconstructed from mtDNA may sometimes be in error because of introgression of these independently segregating organelles from one species to another.

The western Palearctic water frogs are of particular interest since at least three groups of hybrid lineages occur among them (15–18). In all three cases, *Rana ridibunda* Pallas 1771 is one of the parental species. *Rana lessonae* Camerano 1882, *Rana perezi* Seoane 1885, and an unnamed taxon resembling *R. lessonae* from peninsular Italy are the other parental species. In these hybrid lineages, the gametes (whether ova or sperm) normally contain only an intact ridibunda genome; the non-ridibunda genome is excluded from the gametes (19–21). Hybrids are reformed each generation because the hybrids normally mate with the non-ridibunda parental species. Such hybrids, in which one genome is transmitted clonally from generation to generation, while the other genome is newly introduced in each generation, are termed hybridogenetic (22).

We compared the restriction fragment patterns of mtDNA from *R. ridibunda* and *R. lessonae*, the parental species of a number of hybridogenetic lineages that occur throughout much of Central Europe and are collectively known as *Rana esculenta*.

MATERIALS AND METHODS

Specimens. Most samples of frogs were collected from six localities in western Poland, within a 40-km radius of Poznan. In addition, single individuals of *R. ridibunda* were obtained from Pfynwald, Switzerland, and from Gelibolu, Turkey (Table 1).

Preparation of mtDNA. mtDNA was usually isolated from mature ova of females (23), although occasionally liver, heart, and kidney were used instead. Mitochondria were isolated by homogenization of tissue in STE buffer (0.25 M sucrose/0.03 M Tris·HCl/0.01 M EDTA, pH 7.6) and differential centrifugation. Crude mtDNAs were prepared following the procedures outlined by Davis *et al.* (24). In some cases the mitochondrial fraction was banded on a sucrose step gradient (0.9 M and 1.8 M sucrose) prior to lysis.

Crude mtDNA preparations were purified by sequential extractions with phenol and chloroform. mtDNA was precipitated with 2 vol of ethanol, resuspended in TE buffer (10 mM Tris·HCl/1.0 mM EDTA, pH 8.0)/0.25 M sodium acetate, treated with 50 μg of RNase A per ml and 200 units of T1 RNase per ml for 1 hr at 37°C, and then treated with 100 μg of proteinase K per ml for 1 hr at 37°C. This was followed again by organic extractions and ethanol precipitation. Purified mtDNAs were stored at −70°C in a 1:10 dilution of TE buffer. The purity and quantity of each preparation were determined by minigel electrophoresis of a 1–5% aliquot.

Table 1. Collection localities and mtDNA phenotypes

Species	*n*	Locality	mtDNA phenotype A	B	C
R. ridibunda	4	PL: Poznan	0	4	0
	16	PL: Fabianowo*	9	7	0
	5	PL: Naramowice*	2	3	0
	10	PL: Dymaczewo	9	1	0
	2	PL: Lodz near Mosina	2	0	0
	1	CH: Pfynwald	0	1	0
	1	TR: Gelibolu	1	0	0
Total	39		23	16	0
R. lessonae	3	PL: Lodz near Mosina	0	0	3
	27	PL: Naramowice*	0	0	27
	2	PL: Edwardowo*	0	0	2
Total	32		0	0	32

PL, Poland; CH, Switzerland; TR, Turkey.
*Suburb of Poznan.

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Restriction Endonuclease Analysis of mtDNA. Five to 10 ng of each DNA sample were digested to completion with each restriction enzyme by using conditions recommended by the supplier (New England Biolabs or Bethesda Research Laboratories). Resulting DNA fragments were end-labeled with the appropriate α-^{32}P-labeled triphosphate deoxynucleoside and separated according to size by electrophoresis through 1% agarose or 3.5% polyacrylamide gels; separated fragments were detected by autoradiography (11). For each gel, fragment sizes were estimated from mobilities of size standards; for agarose gels, these were λ and PM2 DNAs, each cut with *Hin*dIII; for polyacrylamide gels, they were ϕX174 cut with *Hin*cII and pBR322 cut with *Alu* I.

Calculation of Sequence Divergence. The amount of sequence divergence was estimated by comparing fragment patterns generated with each enzyme. Fragments were considered to be homologous and shared if they migrated the same distance. For an enzyme that cuts each of two mtDNAs at a single site, the single fragment generated in each case was assumed to be homologous. The proportion of shared fragments was estimated by using Nei and Li's (25) equation 21. The percent sequence divergence, δ, was calculated by using Upholt's (26) formula for p as given by Avise *et al.* (9).

RESULTS

A restriction enzyme survey of the mtDNA present in *R. ridibunda* and *R. lessonae* revealed two very different types of mtDNA in *R. ridibunda*, A and B, and one in *R. lessonae*, C. mtDNAs of a single individual of each mtDNA type—A, B, and C—were cleaved with each of 19 restriction enzymes with hexanucleotide recognition sites (Table 2). The sizes of the fragments obtained with each enzyme sum to ≈19.5 kilobases. Between the two types of mtDNA in *R. ridibunda*, A and B, different fragment patterns were found with all enzymes except for *Mlu* I, which cuts each mtDNA only once (Fig. 1). Equivalent differences in fragment patterns were found between A and C (*R. lessonae*) mtDNAs (Table 2). Fragment patterns of B mtDNA, however, were surprisingly similar to those of C mtDNA; with the 19 enzymes used, only *Bam*HI and *Sma* I produced different fragment patterns for the two mtDNAs (Fig. 2).

Similarities in the mtDNAs were calculated for each of the three pairs (Table 3). For the two mtDNA types in *R. ridi-*

Table 2. Restriction fragment patterns in the mtDNAs of *R. ridibunda* (types A and B) and *R. lessonae* (type C)

Enzyme	n_a	A	n_b	B	n_c	C	n_{ab}	n_{ac}	n_{bc}
Ava I	7	7,000	6	7,000	6	7,000	1	1	6
		4,000		6,000		6,000			
		3,200		5,100		5,100			
		2,000		1,100		1,100			
		1,800		130		130			
		1,350		105		105			
		100							
*Bam*HI	1	19,500	3	8,000	3	11,000	0	0	1
				6,400		8,000			
				5,600		470			
Bcl I	3	11,500	4	8,400	4	8,400	1	1	4
		5,150		7,800		7,800			
		2,150		2,150		2,150			
				900		900			
Bgl II	6	10,500	6	5,700	6	5,700	2	2	6
		3,600		3,750		3,750			
		2,150		3,600		3,600			
		1,650		3,000		3,000			
		1,600		1,650		1,650			
		165		1,500		1,500			
*Eco*RI	1	19,500	2	15,000	2	15,000	0	0	2
				4,400		4,400			
*Eco*RV	2	11,000	2	16,000	2	16,000	0	0	2
		8,300		3,500		3,500			
Hae II	4	8,800	5	8,500	5	8,500	1	1	5
		5,400		5,400		5,400			
		3,800		4,200		4,200			
		1,050		625		625			
				500		500			
*Hin*cII	11	5,600	10	5,600	10	5,600	2	2	10
		3,200		3,300		3,300			
		2,900		3,200		3,200			
		2,350		1,900		1,900			
		2,100		1,700		1,700			
		1,200		1,300		1,300			
		630		1,050		1,050			
		500		830		830			
		375		340		340			
		270		110		110			
		195							
*Hin*dIII	7	5,700	8	5,700	8	5,700	4	4	8
		4,500		4,000		4,000			
		4,100		4,000		4,000			
		2,150		2,150		2,150			
		1,200		1,500		1,500			
		1,100		1,100		1,100			
		600		600		600			
				285		285			
Hpa I	4	6,000	3	10,500	3	10,500	2	2	3
		5,200		6,000		6,000			
		5,200		3,250		3,250			
		3,250							
Kpn I	3	13,500	5	8,800	5	8,800	2	2	5
		5,400		5,400		5,400			
		620		3,300		3,300			
				1,500		1,500			
				620		620			
Mlu I	1	19,500	1	19,500	1	19,500	1	1	1
Pst I	3	7,100	2	12,500	2	12,500	1	1	2
		6,100		7,100		7,100			
		6,100							
Pvu II	4	9,300	5	15,000	5	15,000	0	0	5
		5,800		2,250		2,250			
		3,500		1,600		1,600			
		900		750		750			
				270		270			
Sal I	1	19,500	0	No cuts	0	No cuts	0	0	0
Sma I	3	8,800	2	14,000	1	19,500	0	0	0
		8,800		5,500					
		1,800							
Sst I	2	15,500	1	19,500	1	19,500	0	0	1
		4,200							
Xba I	3	11,000	1	19,500	1	19,500	0	0	1
		5,900							
		2,300							
Xho I	1	19,500	0	No cuts	0	No cuts	0	0	0
Total	67		66		65		17	17	62

n_{ab}, number of fragments shared by *R. ridibunda* (A and B) mtDNAs; n_{ac}, number of fragments shared by *R. ridibunda* (A) and *R. lessonae* (C) mtDNAs; n_{bc}, number of fragments shared by *R. ridibunda* (B) and *R. lessonae* (C) mtDNAs. n_a, n_b, and n_c are the number of fragments of types A, B, and C, respectively. The sizes of fragments are given in bases.

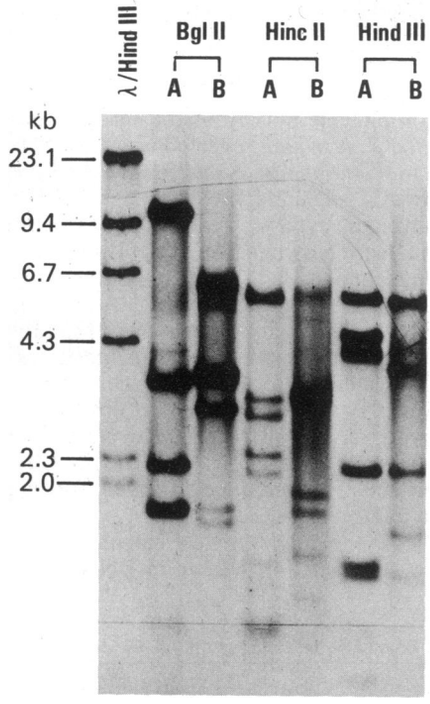

FIG. 1: Autoradiogram of fragments produced by restriction enzyme cleavage of type A and B mtDNAs of *R. ridibunda*. Digests produced by *Bgl* II, *Hinc*II, and *Hind*III were end-labeled with ^{32}P and electrophoresed through a 1% agarose slab gel. The size markers [shown in kilobases (kb)] in the leftmost lane were fragments of λ DNA generated by digestion with *Hind*III.

bunda, A and B, 17 of 133 fragments (25.6%) were shared. This corresponds to a sequence difference of 8.2%. This is similar to the amount of difference, 8.1%, between the A

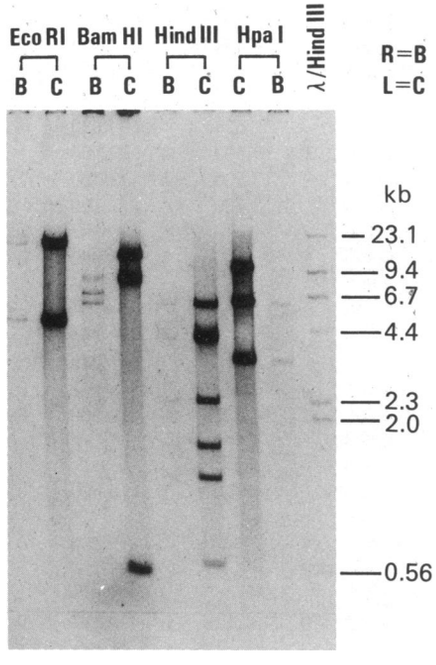

FIG. 2. Autoradiogram of fragments produced by cleavage of *R. ridibunda* type B and *R. lessonae* type C mtDNAs by *Eco*RI, *Bam*HI, *Hind*III, and *Hpa* I. Only *Bam*HI generates different fragment patterns between B and C mtDNAs. The faint band at 10,500 base pairs generated by *Hpa* I digestion of B mtDNA is clearly visible in the original autoradiogram. kb, Kilobases.

Table 3. Quantitative comparison of fragment patterns

mtDNA pair	F	δ
A–B	0.256	8.2 ± 1.0%
A–C	0.258	8.1 ± 1.0%
B–C	0.947	0.3 ± 0.3%

F, proportion of fragments shared by a pair of mtDNAs; δ, percentage difference in sequence between two mtDNAs.

mtDNA of *R. ridibunda* and the C mtDNA of *R. lessonae*. B mtDNA of *R. ridibunda*, in contrast, had almost 95% of its fragments in common with C mtDNA, resulting in a sequence divergence of only 0.3%.

Given the similarity of B mtDNA of *R. ridibunda* to the C type of *R. lessonae*, all *R. ridibunda* and *R. lessonae* mtDNA samples were digested with *Bam*HI and *Sma* I, the only two enzymes that distinguish between all three forms (Table 2). Of the 37 *R. ridibunda* from Poland, 59% had type A mtDNA, whereas 41% had type B (Table 1). The single *R. ridibunda* collected in Switzerland (introduced, probably from southeastern Europe) had the B phenotype, although with a variant shorter mitochondrial genome. By using three enzymes, the single *R. ridibunda* mtDNA sample from Anatolian Turkey was found to have the A phenotype. All 32 *R. lessonae* from Poland had type C mtDNA.

To confirm the phenotypes indicated by *Bam*HI and *Sma* I, a number of mtDNA samples were digested with additional enzymes that produced many fragments and many differences between A and C mtDNAs. Including the single preparations of each type cleaved with all 19 enzymes, 50% of type A, 44% of type B, and 30% of type C mtDNAs were cleaved with more than the two diagnostic enzymes: 8 preparations of type A mtDNA, 4 of type B, and 4 of type C were each cleaved with 3–5 enzymes, whereas 2 of type A, 2 of type B, and 4 of type C were each cut with 7–12 enzymes. In sum, the 71 preparations were cleaved a total of 281 times. Within each mtDNA type, no variation in fragment pattern was found with any of the restriction enzymes; significant variation within each type was found, however, in the total lengths of the mitochondrial genomes (unpublished data).

DISCUSSION

Two strikingly different types of mtDNA occur within *R. ridibunda*. One type, A, apparently the authentic *R. ridibunda* mitochondrial genome, differs from *R. lessonae* (C) mtDNA by 8% of its nucleotides. The other form, B, which we call "*lessonae*-like," differs from *R. lessonae* mtDNA by only 0.3%. The most plausible explanation for the presence of these two types of mtDNA in *R. ridibunda* is an interspecific transfer of mtDNA from *R. lessonae* to *R. ridibunda*. The presence in Turkish *R. ridibunda*, far from the present range of *R. lessonae*, of type A mtDNA is consistent with our identification of this form as that typical of *R. ridibunda*.

As in the other well-documented case of interspecies transfer of mtDNA, between *Mus domesticus* and *Mus musculus* (27), *R. ridibunda* and *R. lessonae* form interspecies hybrids. A less convincing case of interspecies transfer of mtDNA has been reported in a pair of hybridizing *Drosophila* (28). The situation in the frogs is more complicated in that the hybrid forms a series of semi-independent hemiclonal lineages known collectively as *R. esculenta*, which in Central Europe usually produces gametes containing only the *R. ridibunda* chromosome set. *R. esculenta* is maintained by matings with *R. lessonae*; the occasional matings of *R. esculenta* with itself produce *R. ridibunda* progeny.

Transfer of mtDNA from *R. lessonae* to *R. ridibunda* probably occurred not directly, but through an intermediate stage involving the hybrid *R. esculenta*. Transfer through *R. esculenta* may have occurred in two possible ways. One way

is by crosses of *R. esculenta* with *R. esculenta*: since most *R. esculenta* lineages in Central Europe have *R. lessonae* mtDNA (unpublished data), an *R. ridibunda* produced from an *R. esculenta* × *R. esculenta* cross would also probably have *R. lessonae* mtDNA. In the laboratory, these crosses are rarely successful: most embryos do not reach tadpole stage, and very few tadpoles complete metamorphosis (15, 29). Since the clonally inherited *R. ridibunda* genome in *R. esculenta* does not recombine with the *R. lessonae* genome, it can gradually accumulate deleterious recessive mutations (30) that are masked in effect by the *R. lessonae* genome (cf. refs. 31 and 32). In *R. esculenta* × *R. esculenta* crosses, however, deleterious recessive alleles in the clonal *R. ridibunda* genome would be unmasked.

A second, and probably more successful, way to transfer *R. lessonae* mtDNA to *R. ridibunda* is via a cross between an *R. esculenta* female and an *R. ridibunda* male, which would also produce *R. ridibunda* progeny with *R. lessonae* mtDNA.

Although our sampling of *R. ridibunda* and *R. lessonae* was geographically limited, these samples provide no evidence for ongoing introgression of *R. lessonae* mitochondrial genomes into *R. ridibunda*. Almost half of the *R. ridibunda* in Central Europe have a *lessonae*-like (B) mitochondrial genome, but none has been found with the *R. lessonae* (C) mtDNA that is present in both *R. esculenta* (unpublished data) and *R. lessonae*; conversely, the B mtDNA has not been found in either *R. esculenta* or *R. lessonae*. This suggests that the introgression of *lessonae*-like mtDNA into *R. ridibunda* occurred either at some time in the past or at some locality other than central Poland. If the introgression occurred at some other locality, it nevertheless happened long enough ago to allow spread of the introgressed mitochondrial genome into central Poland, where we found no *R. ridibunda* with C mtDNA.

Evidence in this study complements evidence, for this species pair, of introgression of nuclear genes as determined by electrophoretic markers (33, 34). It has seemed possible that the presence in Central European *R. ridibunda* of *lessonae*-like nuclear alleles might reflect inheritance of these alleles from a common ancestor, even though the immunologically estimated divergence date makes this seem unlikely (35). The presence in some *R. ridibunda* of a *R. lessonae* mitochondrial genome appears to confirm that introgression rather than common inheritance is the source of *lessonae*-like nuclear alleles in *R. ridibunda*.

The introgression of *R. lessonae* mtDNA into an *R. ridibunda* nuclear background points up clearly the disjunction in inheritance of nuclear and mitochondrial genes. A similar discordancy between mitochondrial genomes and morphological traits was found in *Peromyscus* by Lansman *et al.* (36), although they suggested that the morphology was not a valid indicator of phylogeny in this case. Phylogenies based on single genes, or even groups of genes, reflect only the particular history of that gene or those genes and not necessarily the history of the whole organism. An example in which possibly up to a third of the nuclear genes would indicate a phylogeny different from that based on the remaining two-thirds has been reported recently in another group of western Palearctic water frogs (37).

Studies of the inheritance of mtDNA provide important and otherwise inaccessible information about the evolutionary histories of closely related species. The problem of determining phylogenetic relationships is complex; an adequate understanding requires combining data from a broad spectrum of traits at various levels, from morphological to molecular.

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